VIROLOGY 236, 374–381 (1997)
ARTICLE NO. VY978743

Hui-Lin Wu,* Pei-Jer Chen,† Jung-Jung Mu,† Wei-Kuang Chi,‡ Tso-Liang Kao,† Lih-Hwa Hwang,* and Ding-Shinn Chen*,[1]

*Hepatitis Research Center, National Taiwan University Hospital, †Graduate Institutes of Clinical Medicine and Microbiology, National Taiwan University College of Medicine, and ‡Development Center for Biotechnology, Taipei, Taiwan

Received May 8, 1997; returned to author for revision June 4, 1997; accepted July 22, 1997

Large delta antigen (L-HDAg) of hepatitis delta virus (HDV) and small-form hepatitis B surface antigen (HBsAg) of helper hepatitis B virus have previously been shown to be the minimum components for the assembly of HDV-like particles in mammalian cells. Extending from this finding, we coexpressed L-HDAg and small HBsAg in *Saccharomyces cerevisiae* to study their assembly in yeast cells. The assembly of virus particles from L-HDAg and HBsAg in yeast was demonstrated by their coexistence in the same isopycnic fractions and by the coimmunoprecipitation of L-HDAg with HBsAg using an antibody against HBsAg (anti-HBs). Furthermore, after purification by affinity chromatography with anti-HBs, HDV-like particles with size and morphology similar to those derived from mammalian cells could be visualized by electron microscopy. Mice immunized with yeast-derived HDV-like particles simultaneously acquired antibodies against HBsAg and HDAg, indicating that both viral proteins are antigenic. The results indicated that *S. cerevisiae* could serve as a host for the assembly of HDV-like empty particles. This system may be useful in investigating cellular processes involved in HDV assembly and in producing ample amount of HDV-like particles for structural and immunological studies.

INTRODUCTION

Hepatitis delta virus (HDV) is a defective human pathogen causing severe acute liver inflammation or progressive chronic liver diseases (Rizzetto and Verme, 1985). It requires a hepadnavirus helper, the human hepatitis B virus (HBV) or the woodchuck hepatitis B virus, to provide envelope proteins for virion assembly and transmission (Rizzetto *et al.,* 1980; Ponzetto *et al.,* 1984).

The virion of HDV is a 36-nm particle, with an envelope composed of lipid coat and three forms of hepatitis B virus surface antigen (HBsAg) (Bonino *et al.,* 1986; He *et al.,* 1989), termed large, middle, and small HBsAg according to their protein sizes (Ganem and Varmus, 1987). Inside the envelope is the 1.7-kb circular RNA genome and two forms of HDV-specific proteins, namely the small (24 kDa) and the large (27 kDa) delta antigens (S- and L-HDAg) (Bonino *et al.,* 1986; Kos *et al.,* 1986; Wang *et al.,* 1986). L-HDAg is identical to S-HDAg except that it contains a 19-amino-acid extension at the carboxyl terminus as a result of an RNA editing event which eliminates the termination codon of S-HDAg (Luo *et al.,* 1990). Although sharing similar biochemical properties, these two proteins play different roles in the viral life cycle. S-HDAg is required for HDV RNA replication (Kuo *et al.,* 1989), whereas L-HDAg inhibits the replication and is essential for virion assembly (Chao *et al.,* 1990; Chang *et al.,* 1991).

Since the host range of hepadnaviruses, and hence the HDV, is very restricted, tissue culture cells have been a convenient system for the study of the assembly of HDV. Cotransfection of HBV- and HDV-expressing plasmids into mammalian cell lines has yielded HDV particles *in vitro* (Wu *et al.,* 1991; Ryu *et al.,* 1992). Furthermore, L-HDAg along with the small form of HBsAg is necessary and sufficient for the assembly of HDV-like particles devoid of viral RNA (Chang *et al.,* 1991; Wang *et al.,* 1991; Ryu *et al.,* 1992). On the other hand, S-HDAg alone can not interact with HBsAg and thus will not be packaged into particles. Nevertheless, it can be copackaged through protein–protein interactions with L-HDAg (Chen *et al.,* 1992).

Although HDV can be assembled in mammalian cells, mass production of HDV particles using this system is not practical. Since HBsAg particles can be assembled in yeast (Valenzuela *et al.,* 1982; Hitzeman *et al.,* 1983) but not in bacteria (Edman *et al.,* 1981; Mackay *et al.,* 1981), we explored the possibility of using yeast, *Saccharomyces cerevisiae,* as an alternative host for the assembly of HDV-like particles. The yeast expression system has been used successfully not only for the assembly of HBsAg particles but also for that of the HBV-like particles (Shiosaki *et al.,* 1991) and the L1, L2-containing particles of human papillomavirus (Hofmann *et al.,* 1995). Importantly, yeast cells have appropriate machineries to carry out posttranslational modifications which may be required for producing biologically active proteins. Estab-

[1] To whom correspondence and reprint requests should be addressed at the Hepatitis Research Center, National Taiwan University Hospital, 7 Chung-Shan South Road, Taipei, Taiwan. Fax: 886-2-3317624 or 3825962.

0042-6822/97 $25.00

lishment of yeast as an alternative host for the assembly of HDV-like particles would make large-scale production of these particles possible. These particles can then serve many purposes, such as immunological and structural studies.

In this communication, we report the coexpression of L-HDAg and HBsAg in yeast and the assembly of these two proteins into HDV-like particles as characterized by isopycnic centrifugation, coimmunoprecipitation, and electron microscopy.

MATERIALS AND METHODS

Strains

Escherichia coli DH5α was used for all bacterial transformations. Yeast strain HF7C (MATa, *ura* 3-52, *his* 3-100, *ade* 2-101, *lys* 2-801, *trp* 1-901, *leu* 2-3, 112, can^r, *gal* 4-542, *gal* 80-538, URA 3::GAL 1-lac Z) was purchased from Clontech (Palo Alto, CA) and used as a host cell for the expression of viral proteins.

Plasmids

Plasmid pSVDAg-L (Chang *et al.,* 1991) is a mammalian vector expressing L-HDAg. pSX-N is a derivative of pDPSX (Wang *et al.,* 1991). The consensus motif for N-linked glycosylation in pSX-N has been mutated, so that it expresses only the unglycosylated form of small HBsAg in mammalian cells (Wang *et al.,* 1996).

To construct and express proteins of interest in yeast, two cloning vectors, pGPD (for HBsAg) and pGAD424 (Clontech) (for S-HDAg and L-HDAg) were used. They are yeast *S. cerevisiae*–*E. coli* shuttle vectors containing promoters of yeast genes (the glyceraldehyde-3-phosphate dehydrogenase gene promoter in pGPD and the alcohol dehydrogenase gene promoter in pGAD424). They also carry different nutritional genes (TRP 1 in pGPD and LEU 2 in pGAD424) that allow yeast auxotrophs to grow on synthetic media lacking tryptophan or leucine. For the construction of pGPD/S (Fig. 1A) expressing small HBsAg in yeast, a 962-bp *Eco*RI–*Hpa*I fragment of HBV DNA was blunted with the Klenow fragment of DNA polymerase I and subcloned into pGPD that had been treated with *Xho*I plus *Eco*RI and blunted with the Klenow fragment. To express S-HDAg and L-HDAg, DNA fragments containing S- or L-HDAg open reading frame were generated from pSVDAg-S (Kuo *et al.,* 1989) or pSVDAg-L (Chang *et al.,* 1991) by either *Sca*I plus *Sal*I or *Sca*I plus *Pst*I digestion. Both fragments were blunted and subcloned into a modified pGAD424 from which the 680-bp *Hin*dIII–*Hin*dIII fragment had been deleted to remove the GAL4-DNA-activation domain, and the resultant ends were filled in with the Klenow fragment. The resulting plasmids were designated pGAD/S-HDAg and pGAD/L-HDAg, respectively (Fig. 1A).

Transfection of DNA

Transfection of HuH-7 cells was performed by the calcium phosphate precipitation method as described previously (Wu *et al.,* 1991).

Transformation of plasmid DNA into yeast cells

Plasmid DNA was introduced into yeast using lithium chloride according to the methods developed previously with some modifications (Gietz *et al.,* 1992). To coexpress two proteins in a yeast cell, pGAD/L-HDAg or pGAD/S-HDAg was cotransformed with pGPD/S into yeast cells and the cotransformants were selected in SD synthetic medium lacking leucine and tryptophan.

Preparation of yeast extracts

Transformed yeast cells were first cultured in Leu$^-$Trp$^-$ SD synthetic medium overnight, transferred to 10-fold volume of YEPD medium (1% yeast extract, 2% peptone, 2% glucose), and cultured at 30° to midlog phase or for about 20 hr for large-scale preparation. Cells were collected by centrifugation (2000 *g,* 5 min), resuspended in P_i/NaCl (10 m*M* sodium phosphate, 0.85% NaCl) with 0.1% Triton X-100, and broken by 1 min of vigorous vortexing with glass beads (0.45–0.60 mm; Sigma, St. Louis, MO) four times with intermittent cooling on ice or in Dyno Mill KDL (Batch) (3000 rpm, 8 min). The lysates were clarified by centrifugation at 10,000 *g* for 10 min at 4° and the supernatants were collected for analysis or further purification.

Immunoblot analysis and immunoprecipitation of proteins

Aliquots of protein extracts from transformed cells were subjected to electrophoresis on 12% polyacrylamide gels. The gels were either stained by BM fast staining (Boehringer Mannheim) for total protein detection or transferred electrophoretically to a nitrocellulose filter (Hybond-C; Amersham, Buckinghamshire, UK) and subjected to immunoblotting as described before (Chang *et al.,* 1991; Wang *et al.,* 1996).

Immunoprecipitations were performed with either anti-HBs (1:300 dilution) or anti-HDAg (1:500). Transformed yeast cells were grown and disrupted, as described in the previous section, in immunoprecipitation buffer (50 m*M* Tris–HCl, pH 8.0, 150 m*M* NaCl, 5 m*M* EDTA, and 0.1% Triton X-100). Protein extracts were incubated with the specific antibody for 60 min, followed by addition of 30 μl of a 50% protein G-conjugated agarose for another 60 min. The precipitate was washed three times with the same buffer and antigen–antibody complexes were eluted from the agarose beads by boiling in 1$\times$ Laemmli loading buffer (0.0625 *M* Tris–HCl, pH 6.8, 2% SDS, 10% v/v glycerol, 0.1 *M* DTT) for 5 min. The eluted products were then examined by immunoblotting with anti-HDAg or anti-HBs antibody.

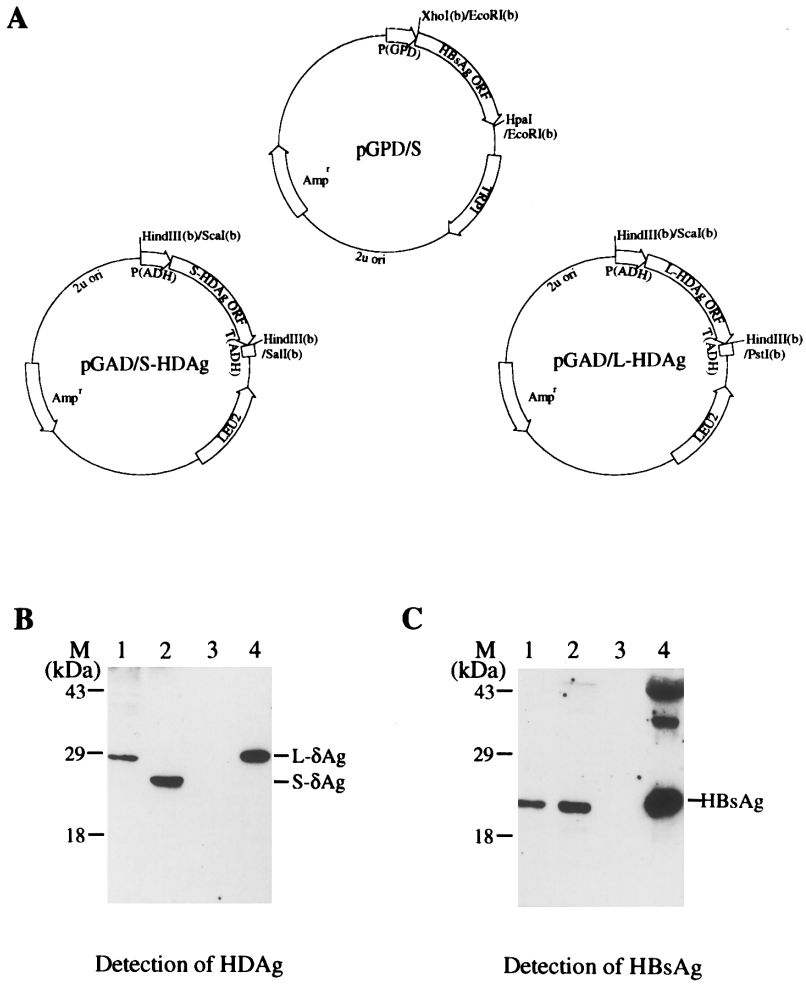

FIG. 1. (A) Plasmid maps of pGPD/S, pGAD/S-HDAg, and pGAD/L-HDAg for the expression of HBsAg, L-HDAg, and S-HDAg, respectively, in yeast. P(ADH) and T(ADH) represent the promoter and terminator of the yeast alcohol dehydrogenase gene. P(GPD) represents the promoter of the glyceraldehyde-3-phosphate dehydrogenase gene. Letter "b" in parentheses indicates that the restriction enzyme-digested ends were blunted with Klenow enzyme. (B and C) Immunoblotting analysis of HDAg and HBsAg in transformed yeast cells. 50 μg of total proteins from transformed yeast cells or transfected HuH-7 cells was subjected to immunoblotting analysis with either anti-HDAg antiserum (B) or anti-HBsAg antibody (C). Lanes 1, 2, and 3 were protein extracts from cells cotransformed with pGPD/S and pGAD/L-HDAg (lane 1), pGPD/S and pGAD/S-HDAg (lane 2), and untransformed cells (lane 3), respectively. Lane 4 was the cell lysate of HuH-7 cells cotransfected with pSVDAg-L and pSX-N.

Cesium chloride gradient analysis of yeast lysates

The clarified lysates of transformed cells were layered on a 20% sucrose cushion and then centrifuged in an SW 28 (Model L-8 M; Beckman) rotor at 25,000 rpm for 16 hr. The pellet was dissolved in 0.5 ml P_i/NaCl, layered onto a discontinuous cesium chloride gradient (1.1–1.4 g/cm^3), and centrifuged at 35,000 rpm in an SW 41 rotor for 24 hr. Fractions of 0.5 ml were collected from the bottom, diluted with five volumes of P_i/NaCl, and centrifuged in a TLA 100.3 rotor (TL-100; Beckman) at 60,000 rpm for 2 hr, and the resulting pellets were resuspended in P_i/NaCl. The presence of HBsAg and HDAg in each fraction was then assayed by immunoblotting.

Affinity chromatography and electron microscopy

The clarified protein extract of transformed cells or culture supernatant from transfected HuH-7 cells was centrifuged in an SW 28 rotor at 25,000 rpm, 4° for 16 hr. The pellets were resuspended in 100 m*M* phosphate buffer (pH 8.0) and then loaded onto a column of Sepharose 4B conjugated with mouse anti-HBs antibody. The absorbed antigens (and particles) were eluted with 3 *M* sodium thiocyanate/0.1 *M* sodium chloride, then desalted and concentrated by a Centricon-30 (Amicon, Beverly, MA).

Samples purified from affinity chromatography were negatively stained with 2% potassium phosphotungstic acid and examined by electron microscopy (Hitachi, H7100).

Expression and purification of recombinant S-HDAg in *E. coli*

The HDV cDNA fragment from nucleotides 1598–1012 was amplified by polymerase chain reaction and sub-

cloned into a pET15a vector (Novagen, Madison, WI). The resulting plasmid was transformed into *E. coli* BL21(DE3) to express a recombinant S-HDAg having N-terminal His-Tag by induction with 1 m*M* isopropyl-β-D-thiogalactopyranoside. The recombinant S-HDAg was purified by affinity chromatography with a nickel column (Novagen) and then continuous elution gel electrophoresis (Prep Cell, Model 491; Bio-Rad, Hercules, CA) as described in the manufacturer's instructions.

Immunization of mice and assay of antibodies

Six-week-old female Balb/c mice were injected with one of the following antigens: (i) 20 μg protein extract from untransformed yeast cells; (ii) affinity-purified HDV-like empty particles in different amounts: one contained 400 ng HBsAg and 20 ng L-HDAg and the other contained 200 ng HBsAg and 10 ng L-HDAg; or (iii) 1 μg purified recombinant S-HDAg derived from *E. coli.* Antigens were emulsified with complete Freund's adjuvant and inoculated into the mice intraperitoneally. The mice were boosted once with the same antigen preparations in incomplete Freund's adjuvant 4 weeks later. Serum specimens were collected on day 14 after the booster and assayed for specific antibodies.

The murine serum specimens were diluted in 1:100 to detect anti-HBs by a commercial enzyme immunoassay kit (Ausab EIA Diagnostic Kit; Abbott Laboratories, North Chicago, IL). Antibody to HDAg was assayed by enzyme-linked immunosorbent assay (ELISA) with purified HDAg following standard procedures. Recombinant S-HDAg purified from *E. coli* was adsorbed to wells of a microtiter plate at a concentration of 5 μg/ml (100 μl/well) in carbonate coating buffer (60 m*M* Na_2CO_3, 35 m*M* $NaHCO_3$, pH 9.6) at 4° overnight. Mouse sera at 1:100 dilution were added to the wells and incubated at 37° for 2 hr. For color development, goat anti-mouse antibody conjugated with horseradish peroxidase (Amersham) at 1:2000 and the substrate (*o*-phenylenediamine and H_2O_2) were added sequentially to the plates. Colorimetric quantitation was then performed with a ELISA reader (Model Σ 960; Metertech, Taipei).

RESULTS AND DISCUSSION

Coexpression of HBsAg and L-HDAg or S-HDAg in yeast cells

To examine whether yeast *S. cerevisiae* can serve as an alternative host for HDV assembly, we first coexpressed L-HDAg and small HBsAg in yeast cells. Yeast cells were cotransformed with either pGPD/S and pGAD/L-HDAg or pGPD/S and pGAD/S-HDAg (as a negative control for the assembly) and selected in synthetic media lacking tryptophan and leucine for cotransformants. Lysates from cells growing on selective media were then assayed for the presence of HBsAg and HDAg by immunoblot analysis. Both L-HDAg (Fig. 1B, lane 1) and HBsAg

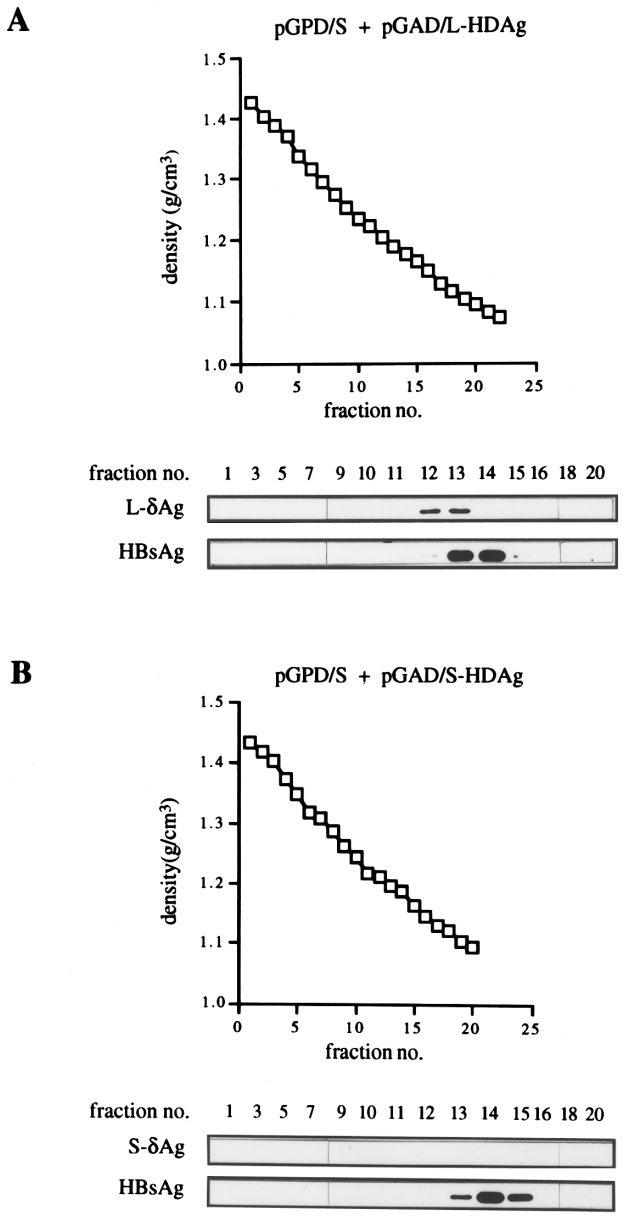

FIG. 2. Cesium chloride gradient centrifugation analysis of the lysates from yeast cells cotransformed with either pGPD/S and pGAD/L-HDAg (A) or pGPD/S and pGAD/S-HDAg (B). The profile of density (top) and results of immunoblotting analysis of HDAg and HBsAg in each fraction (bottom) are shown. The number of each lane represents the fraction number of the CsCl density equilibrium centrifugation.

(Fig. 1C, lane 1) or S-HDAg and HBsAg (lanes 2 in Figs. 1B and 1C) were expressed in cotransformed cells. Consistent with previous reports, only a single band corresponding to the size of unglycosylated small HBsAg was detected (Valenzuela *et al.,* 1982; Hitzeman *et al.,* 1983), indicating that most molecules of the small HBsAg in yeast were not glycosylated. No signal was detected in extracts derived from untransformed yeast cells (lanes 3, Figs. 1B and 1C). For comparison, lysates from HuH-7 cells cotransfected with plasmids expressing L-HDAg (pSVLDAg-L) and unglycosylated form of small HBsAg (SX-N) were also analyzed (lanes 4 in Figs. 1B and 1C).

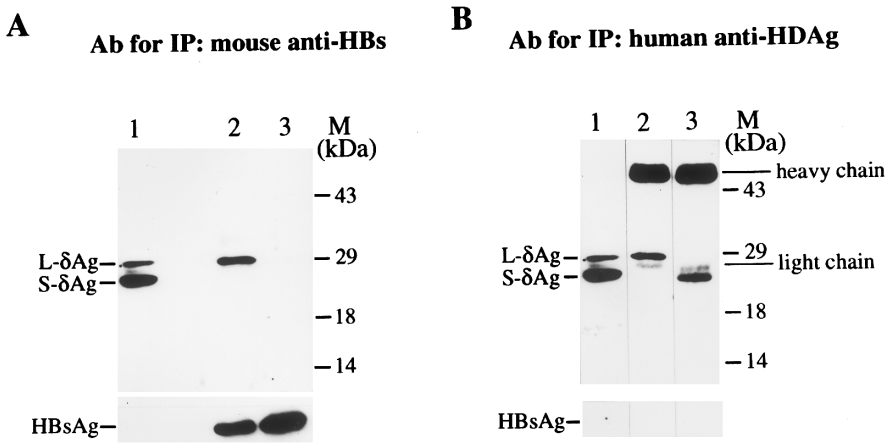

FIG. 3. Immunoprecipitation of yeast lysates with anti-HBs (A) or anti-HDAg antiserum (B). The crude extracts of transformed yeast cells were immunoprecipitated with specific antibodies and the immunoprecipitates were subjected to immunoblotting analysis with human anti-HDAg antibody (top) or mouse anti-HBs antibody (bottom). Lanes 1, L- and S-HDAgs from HuH-7 cells as a positive control. Lanes 2, immunoprecipitates from yeast cells cotransformed with pGPD/S and pGAD/L-HDAg. Lanes 3, immunoprecipitates from yeast cells cotransformed with pGPD/S and pGAD/S-HDAg. Heavy chain and light chain in B represent the heavy and light chains of human immunoglobulins from human anti-HDAg antibody in immunoprecipitation.

The viral proteins expressed in yeast cells have mobility in SDS–PAGE gels similar to that of those expressed in mammalian cells. The yield of HBsAg was estimated by ELISA (Auszyme, Abbott) and that of HDAg by Western blot. The amounts of HBsAg and L-HDAg were about 0.1 and 0.01–0.05%, respectively, of total cellular proteins. The result of HBsAg was comparable to those of other studies (Valenzuela *et al.,* 1982; Hitzeman *et al.,* 1983).

Characterization and electron microscopic study of particles purified from yeast

To examine whether HBsAg and L-HDAg can be assembled into HDV-like empty particles in yeast, crude extracts from cotransformed yeast cells were subjected to CsCl equilibrium centrifugation. After centrifugation, fractions were collected and each was assayed for the presence of specific proteins by immunoblotting. As shown in Fig. 2A, in the extracts from cells cotransformed with pGPD/S and pGAD/L-HDAg, HBsAg and L-HDAg were found in the same isopycnic fractions (fractions 12–13) with a density of around 1.20 g/cm^3. It appears that fraction 14 contained only HBsAg, suggesting that most of the particles in this fraction were empty HBsAg particles. The buoyant density of fraction 14 is approximately 1.18 g/cm^3, consistent with the previously reported results of the serum- and yeast-derived empty HBsAg particles (Hitzeman *et al.,* 1983). As a control (Fig. 2B), only HBsAg, and no S-HDAg, could be detected in the isopycnic fractions around 1.20 g/cm^3 in the extracts from cells cotransformed with pGPD/S and pGAD/S-HDAg.

To further demonstrate that L-HDAg is indeed assembled into HBsAg particles in yeast, coimmunoprecipitation assay was performed. Protein extracts from cells cotransformed with either pGPD/S and pGAD/L-HDAg or pGPD/S and pGAD/S-HDAg were first immunoprecipitated with anti-HBs. The immunoprecipitates were then separated by SDS–PAGE and analyzed by immunoblotting. As shown in Fig. 3A, L-HDAg could be coimmunoprecipitated with HBsAg by anti-HBs (lane 2), indicating that L-HDAg could interact with HBsAg to form HDV-like empty particles in the yeast system. As a control, anti-HBs failed to coimmunoprecipitate S-HDAg with HBsAg (lane 3), suggesting that S-HDAg alone was not packaged into HBsAg particles, similar to the finding in the mammalian system. In contrast, HBsAg could not be precipitated with anti-HDAg (Fig. 3B, bottom, lane 2), suggesting that packaged L-HDAg was inside the HBsAg particles and thus could not interact with anti-HDAg antibody. However, L-HDAg from cotransformed yeast cells could still be immunoprecipitated by anti-HDAg antibody (Fig. 3B, top, lane 2), implying that not all L-HDAg was inside the particles. These free forms of L-HDAg may result from the unassembled L-HDAg or from disruption of particles during the protein extraction process.

Isoprenylation of L-HDAg at the C terminus has been shown to be indispensable for the interactions between L-HDAg and HBsAg and hence the formation of HDV particles (Glenn *et al.,* 1992; Hwang and Lai, 1993). Assembly of HDV-like particles in yeast as we have reported here implied that L-HDAg synthesized in yeast was isoprenylated. Yeast farnesyltransferase (FTase) has been known to be biochemically similar to mammalian FTase (Omer and Gibbs, 1994) which farnesylates HDAg *in vitro* and in animal cells (Otto and Casey, 1996). Furthermore, our results also suggested that, like in the case of HBsAg particle formation (Valenzuela *et al.,* 1982), glycosylation of HBsAg is not essential for the formation of HDV-like particles. The observations are consistent with our recent results in human hepatoma cell lines (Wang *et al.,* 1996).

Yeast-derived HBsAg-containing proteins purified by af-

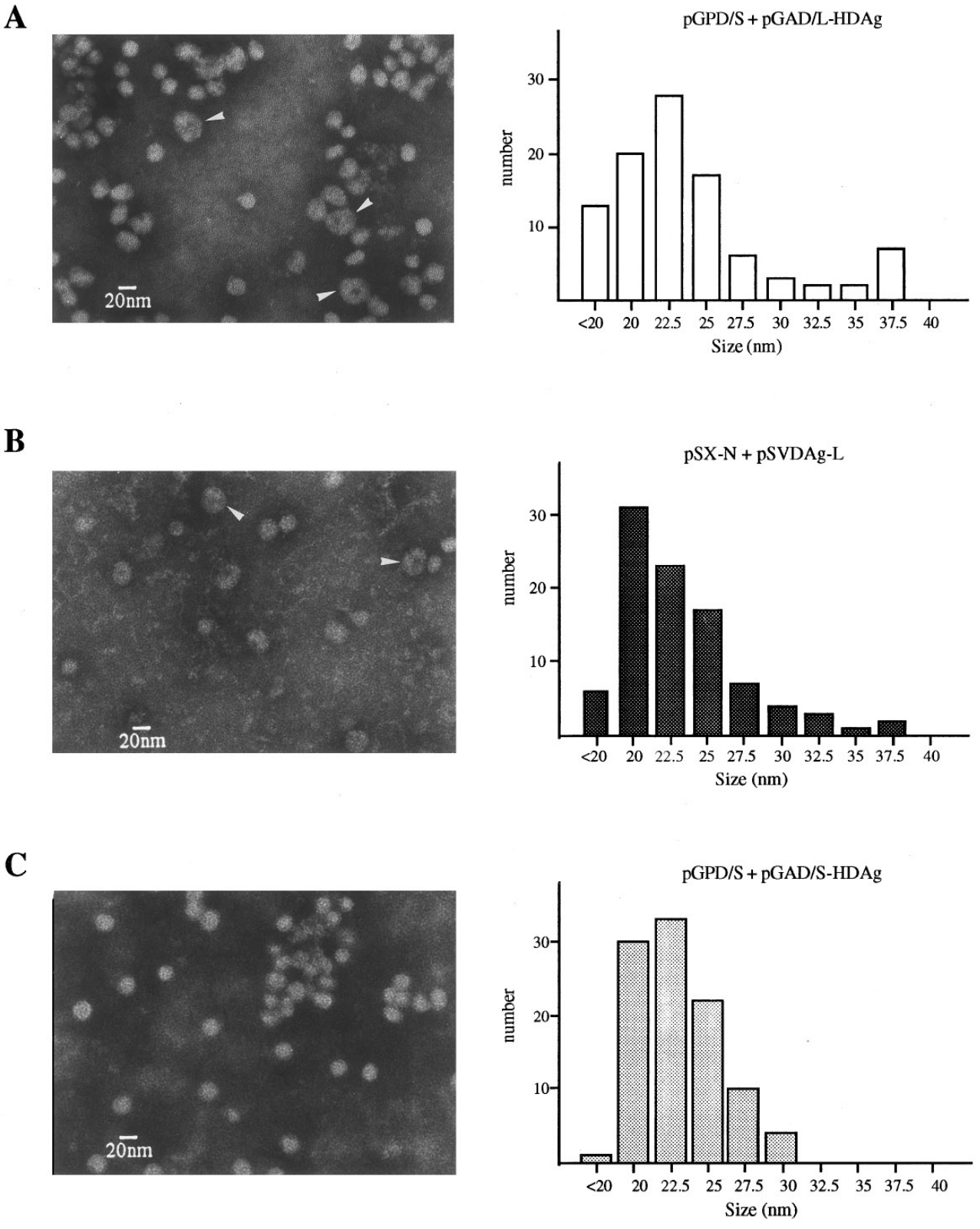

FIG. 4. Electron microscopic examination of HBsAg-containing particles derived from yeast. (Left) Electron micrograph of particles purified by immunoaffinity chromatography from yeast cells cotransformed with pGPD/S and pGAD/L-HDAg (A), culture supernatant of HuH-7 cells cotransfected with pSVDAg-L and pSX-N (B), and yeast cells cotransformed with pGPD/S and pGAD/S-HDAg (C). Purified particles were negatively stained with phosphotungstic acid. Representative particles with a diameter larger than 30 nm are indicated by white arrowheads. (Right) Histograms of the size distribution of particles, which were generated by measuring the diameters of 100 intact particles observed in representative fields. The particle diameter was inferred assuming that the particles were perfect spheres.

finity chromatography of anti-HBs antibody were examined by electron microscopy for HDV-like particles. Particles with diameters ranging from 20 to 37 nm were present in samples purified from yeast cells cotransformed with pGPD/S and pGAD/L-HDAg. Figure 4A shows that these particles could be divided into two populations; the major one is smaller than 30 nm and peaked at 20–25 nm; the other is larger than 30 nm and peaked around 37 nm (arrowheads indicate representative particles). The size distribution of particles purified from yeast was compared to that purified from HuH-7 cells transfected with L-HDAg- and nonglycosylated small HBsAg-expression vectors. The patterns appear to be very similar (compare Figs. 4A and 4B). In contrast, particles purified from pGPD/S- and pGAD/S-HDAg-cotransformed yeast cells, which should consist of HBsAg particles only, revealed only a major population peaking at 20–22 nm; no particles larger than 30 nm were observed (Fig. 4C). The size distribution of these particles is reminiscent of the reported HBsAg particles in yeast (Petre *et al.,* 1987). We presumed that the particles with sizes around 37 nm, similar to the size of the natural virion (He *et al.,* 1989), were HDV-like particles, and those with sizes around 22 nm were most likely HBsAg particles.

Calculating the exact proportion of HDV-like particles in yeast lysates was difficult due to the lack of satisfactory methods to separate HDV-like and HBsAg empty particles and also the uncertainty regarding the molar ratio of HBsAg to L-HDAg in an HDV-like empty particle. However, by electron microscopic observation, HDV-like particles were roughly estimated to constitute about 5–10% of total particles in both yeast and mammalian preparations. By SDS–PAGE analysis, the ratio of L-HDAg to HBsAg in purified particles, as estimated by the signal strength of staining, is 0.12 and 0.1 for yeast- and mammalian cell-derived particles, respectively. The packaging efficiency of HDV-like empty particles in yeast system thus appears to be similar to that in mammalian cells.

Induction of antibodies to both HBsAg and HDAg by HDV-like empty particles from yeast

The immunogenicity of yeast-derived HDV-like empty particles was tested in mice. Sera of the mice immunized with yeast-derived HDV-like particles were demonstrated to contain antibodies against both HDAg and HBsAg by ELISA (Fig. 5). The specificity of these antibodies was confirmed by immunoblot analysis (data not shown). As a control, mice immunized with either purified S-HDAg from *E. coli* lysates or crude extracts of untransformed yeast cells produced antibody against HDAg only or no antibody against HDAg and HBsAg, respectively (Fig. 5). The results indicated that the yeast-derived HDV-like particles were immunogenic in eliciting humoral immunity against both HDAg and HBsAg in mice.

HDAg is the only known protein causing antibody responses after an HDV infection, but the role of this antibody in protecting against HDV is still not clear. Immunizing susceptible woodchucks or chimpanzees with recombinant S-HDAg may limit the level of hepatitis D viremia, but failed to protect the animals (Kos *et al.,* 1991; Karayiannis *et al.,* 1993). However, the recombinant HDAg used in previous studies were all free polypeptides. As intact HBsAg particles have been known to be more immunogenic than free proteins (Cabral *et al.,* 1978), HDAg in particle forms may also yield a better immunogenicity. Our results in mice indicated that the yeast-derived HDV-like particles were quite immunogenic. Thus, the successful assembly of HDV-like particles in yeast may be used for the production of large amounts of these particles for further studies in susceptible animals, such as woodchucks, to examine their immunogenicity and protective efficacy as a potential vaccine.

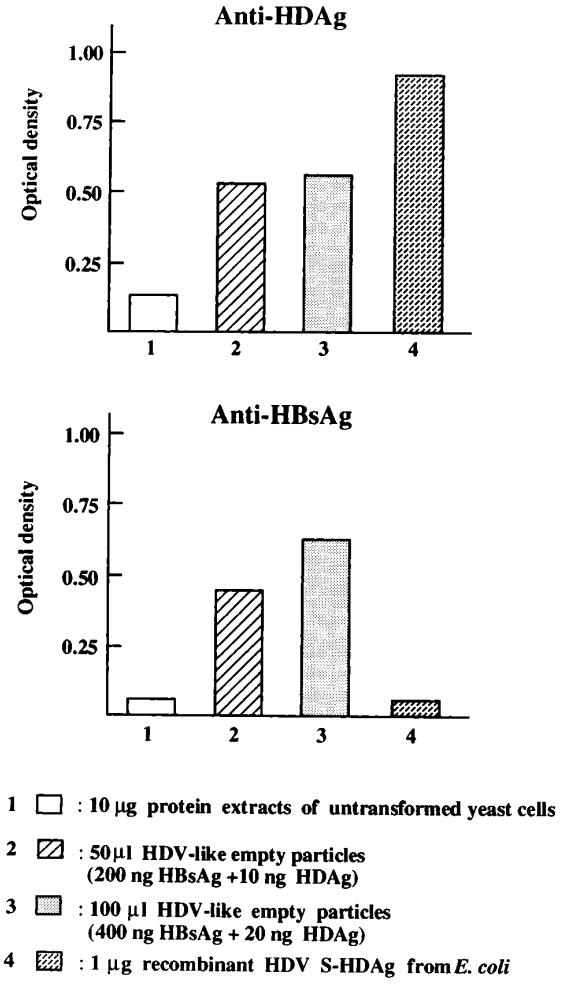

FIG. 5. Detection of HDAg- and HBsAg-specific antibodies in immunized Balb/c mice. Antibodies to HDAg (top) and to HBsAg (bottom) were detected by ELISA as described under Materials and Methods. The *y* axis depicts optical densities of the ELISA.

ACKNOWLEDGMENTS

We thank Dr. S. C. Lee (Graduate Institute of Molecular Medicine, College of Medicine, National Taiwan University, Taipei) for providing

anti-HBsAg antibody ($A_{10}F_1$), Dr. Y. D. Chu (Development Center for Biotechnology, Taipei) and her colleagues for advice and assistance with affinity chromatography, Miss C. Y. Lin and C. S. Zen for electron microscopy, and Ms. I. H. Chiu and H. L. Liu for technical assistance. We also thank Dr. M. L. Kuo, Dr. H. C. Chang, and Ms. S. H. Yeh for critically reading the manuscript. This work was supported by grants from National Health Research Institutes, Taipei, Taiwan.

REFERENCES

Bonino, F., Heermann, K. H., Rizzetto, M., and Gerlich, W. H. (1986). Hepatitis delta virus: Protein composition of delta antigen and its hepatitis B virus-derived envelope. *J. Virol.* 58, 945–950.

Cabral, G. A., Marciano-Cabral, F., Funk, G. A., Sanchez, Y., Hollinger, F. B., Melnick, J. L., and Dreesman, G. R. (1978). Cellular and humoral immunity in guinea pigs to two major polypeptides derived from hepatitis B surface antigen. *J. Gen. Virol.* 38, 339–350.

Chang, F. L., Chen, P. J., Tu, S. J., Wang, C. J., and Chen, D. S. (1991). The large form of hepatitis delta antigen is crucial for assembly of hepatitis delta virus. *Proc. Natl. Acad. Sci. USA* 88, 8490–8494.

Chao, M., Hsieh, S. Y., and Taylor, J. (1990). Role of two forms of hepatitis delta virus antigen: Evidence for a mechanism of self-limiting genome replication. *J. Virol.* 64, 5066–5069.

Chen, P. J., Chang, F. L., Wang, C. J., Lin, C. J., Sung, S. Y., and Chen, D. S. (1992). Functional study of hepatitis delta virus large antigen in packaging and replication inhibition: Role of the amino-terminal leucine zipper. *J. Virol.* 66, 2853–2859.

Edman, J. C., Hallewell, R. A., Valenzuela, P., Goodman, H. M., and Rutter, W. J. (1981). Synthesis of hepatitis B surface and core antigens in *E. coli*. *Nature* 291, 503–506.

Ganem, D., and Varmus, H. E. (1987). The molecular biology of the hepatitis B virus. *Annu. Rev. Biochem.* 56, 651–693.

Gietz, D., St. Jean, A., Woodsand, R. A., and Schiestl, R. H. (1992). Improved method for high efficiency transformation of intact yeast cells. *Nucleic Acids Res.* 20, 1425.

Glenn, J. S., Watson, J. A., Havel, C. M., and White, J. M. (1992). Identification of a prenylation site in delta virus large antigen. *Science* 256, 1331–1333.

He, L. F., Ford, E., Purcell, R. H., London, W. T., Phillips, J., and Gerin, J. L. (1989). The size of hepatitis delta agent. *J. Med. Virol.* 27, 31–33.

Hitzeman, R. A., Chen, C. Y., Hagie, F. E., Patzer, E. J., Liu, C. C., Estell, D. A., Miller, J. V., Yaffe, A., Kleid, D. G., Levinson, A. D., and Oppermann, H. (1983). Expression of hepatitis B virus surface antigen in yeast. *Nucleic Acids Res.* 11, 2745–2763.

Hofmann, K. J., Cook, J. C., Joyce, J. G., Brown, D. R., Schultz, L. D., George, H. A., Rosolowsky, M., Fife, K. H., and Jansen, K. U. (1995). Sequence determination of human papillomavirus type 6a and assembly of virus-like particles in *Saccharomyces cerevisiae*. *Virology* 209, 506–518.

Hwang, S. B., and Lai, M. M. (1993). Isoprenylation mediates direct protein–protein interaction between hepatitis large delta antigen and hepatitis B virus surface antigen. *J. Virol.* 67, 7659–7662.

Karayiannis, P., Saldanha, J., Jackson, A. M., Luther, S., Goldin, S. R., Monjardino, J., and Thomas, H. C. (1993). Partial control of hepatitis delta virus superinfection by immunization of woodchucks (*Marmota monax*) with hepatitis delta antigen expressed by a recombinant vaccinia or baculovirus. *J. Med. Virol.* 42, 210–214.

Kos, A., Dijkema, R., Arnberg, A. C., Van der Meide, P. H., and Schellekens, H. (1986). The hepatitis delta (δ) virus possesses a circular RNA. *Nature (London)* 323, 558–560.

Kos, T., Molijn, A., Blauw, B., and Schellekens, H. (1991). Baculovirus-directed high level expression of the hepatitis delta antigen in *Spodoptera frugiperda* cells. *J. Gen. Virol.* 72, 833–842.

Kuo, M. Y.-P., Chao, M., and Taylor, J. (1989). Initiation of replication of the human hepatitis delta virus genome from cloned DNA: Role of delta antigen. *J. Virol.* 63, 1945–1950.

Luo, G. X., Chao, M., Hsieh, S. Y., Sureau, C., Nishikuraand, K., and Taylor, J. (1990). A specific base transition occurs on replicating hepatitis delta virus RNA. *J. Virol.* 64, 1021–1027.

MacKay, P., Pasek, M., Magazin, M., Kovacic, R. T., Allet, B., Stahl, S., Gilbert, W., Schaller, H., Bruce, S. A., and Murray, K. (1981). Production of immunologically active surface antigens of hepatitis B virus by *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* 78, 4510–4514.

Omer, C. A., and Gibbs, J. B. (1994). Protein prenylation in eukaryotic micro-organisms: Genetics, biology and biochemistry. *Mol. Microbiol.* 11, 219–225.

Otto, J. C., and Casey, P. J. (1996). The hepatitis delta virus large antigen is farnesylated both *in vitro* and in animal cells. *J. Biol. Chem.* 271, 4569–4572.

Petre, J., Van Wijnendaele, F., De Neys, B., Conrath, K., Van Opstal, O., Hauser, P., Rutgers, T., Cabezon, T., Capiau, C., Harford, N., Wilde, M. D., Stephenne, J., Carr, S., Hemling, H., and Swadesh, J. (1987). Development of a hepatitis B vaccine from transformed yeast cells. *Postgrad. Med. J.* 63(Suppl. 2), 73–81.

Ponzetto, A., Cote, P. J., Popper, H., Hoyer, B. H., London, W. T., Ford, E. C., Bonino, F., Purcell, R. H., and Gerin, J. L. (1984). Transmission of hepatitis B-associated delta agent to the eastern woodchuck. *Proc. Natl. Acad. Sci. USA* 81, 2208–2221.

Rizzetto, M., Hoyer, B., Canese, M. G., Shih, J. W.-K., Purcell, R. H., and Gerin, J. L. (1980). Delta agent: Association of δ antigen with hepatitis B surface antigen and RNA in serum of δ-infected chimpanzees. *Proc. Natl. Acad. Sci. USA* 77, 6124–6128.

Rizzetto, M., and Verme, G. (1985). Delta hepatitis: Present status. *J. Hepatol.* 1, 187–193.

Ryu, W. S., Bayer, M., and Taylor, J. (1992). Assembly of hepatitis delta virus particles. *J. Virol.* 66, 2310–2315.

Shiosaki, K., Takata, K., Nishimura, S., Mizokami, H., and Matsubara, K. (1991). Production of hepatitis B virion-like particles in yeast. *Gene* 106, 143–149.

Valenzuela, P., Medina, A., and Rutter, W. J. (1982). Synthesis and assembly of hepatitis B virus surface antigen particles in yeast. *Nature (London)* 298, 347–350.

Wang, C. J., Chen, P. J., Wu, J. C., Patel, D., and Chen, D. S. (1991). Small form hepatitis B surface antigen is sufficient to help in the assembly of hepatitis delta virus-like particles. *J. Virol.* 65, 6630–6636.

Wang, C. J., Sung, S. Y., Chen, D. S., and Chen, P. J. (1996). N-linked glycosylation of hepatitis B surface antigens is involved but not essential in the assembly of hepatitis delta virus. *Virology* 220, 28–36.

Wang, K. S., Choo, Q. L., Weiner, A. J., Ou, J. H., Najarian, R. C., Thayer, R. M., Mullenbach, G. T., Denniston, K. J., Gerin, J. L., and Houghton, M. (1986). Structure, sequence and expression of the hepatitis delta viral genome. *Nature (London)* 323, 508–513.

Wu, J. C., Chen, P. J., Kuo, Y. P., Lee, S. D., Chen, D. S., and Ting, L. P. (1991). Production of hepatitis delta virus and suppression of helper hepatitis B virus in a human hepatoma cell line. *J. Virol.* 65, 1099–1104.